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Functional role of N-linked glycosylation on the rat melanin-concentrating hormone receptor 1

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Abstract **Melanin-concentrating hormone (MCH) is known to act through two G-protein-coupled receptors MCHR1 and MCHR2. MCHR1 has three potential sites (Asn^{13}, Asn^{16} and Asn^{23}) for *N*-linked glycosylation in its extracellular amino-terminus which may modulate its reactivity. Site-directed mutagenesis of the rat MCHR1 cDNA at single or multiple combinations of the three potential glycosylation sites was used to examine the role of the putative carbohydrate chains on receptor activity. It was found that all three potential *N*-linked glycosylation sites in MCHR1 were glycosylated, and that *N*-linked glycosylation of Asn^{23} was necessary for full activity. Furthermore, disruption of all three glycosylation sites impaired proper expression at the cell surface and receptor activity. These data outline the importance of the *N*-linked glycosylation of the MCHR1.**

Key words: Melanin-concentrating hormone; G-protein-coupled receptor; *N*-glycosylation site; Calcium influx; Ligand binding

1. Introduction

Melanin-concentrating hormone (MCH) is a 19-amino acid cyclic peptide that was originally isolated from salmon pituitary glands [1]. The rat counterpart was found in the hypothalamus [2] and expressed mainly in the cell bodies of the lateral hypothalamus and zona incerta with projections widely distributed throughout the central nervous system [3]. In mammals, MCH plays various biological roles, including the central regulation of feeding behavior. Intracerebroventricular administration of MCH promotes feeding behavior in rats [4,5]. Targeted disruption of the MCH gene in mice results in a lean phenotype as a result of hypophagia and increased metabolic rate [5], whereas transgenic mice overexpressing MCH are obese and insulin-resistant [6].

An orphan G-protein-coupled receptor (GPCR), originally called SLC-1 [7], was identified as a cognate receptor of MCH [8–12]. This receptor, presently named MCHR1, is expressed in the brain at high levels [11,13,14] and in other regions at low to moderate levels [11]. Cells transfected with MCHR1 responded to MCH by an increase in intracellular free Ca^{2+} levels, inhibition of forskolin-stimulated cyclic AMP production and activation of mitogen-activated protein kinase [10–12,15]. A second MCH receptor, human MCHR2, was isolated by sequence data mining [16], but several non-human species, including Rodentia, do not have functional MCHR2 receptors [17]. A very recent study showed that MCHR1-deficient mice are lean, hyperactive, and hyperphagic and have altered metabolism [18,19], suggesting that MCHR1 in mice plays a role in energy homeostasis.

Glycosylation is a common posttranslational feature in the GPCR superfamily. It has been shown that glycosylation of the receptor may be involved in a variety of biological activities. These activities include maintenance of receptor stability, folding, trafficking of the receptor to the cell surface, ligand binding, and signal transduction. We decided to analyze whether glycosylation is also important for MCHR1 activity. Western blot analysis of HEK293 cells transfected with MCHR1 exhibited a broad 60-kDa band when MCHR1 is detected with an antiserum against its C-terminus [13]. This molecular weight is higher than that predicted from the MCHR1 cDNA sequence, and may represent a glycosylated form of the receptor. This is supported by the fact that the presumed extracellular amino-terminal part of MCHR1 contains three consensus sequences (Asn^{13}, Asn^{16} and Asn^{23}) that would be suitable for *N*-glycosylation [7].

In this study, we prove that MCHR1 is indeed glycosylated. we also analyze the functional role of the three putative *N*-glycosylation sites of MCHR1 through suppression of *N*-glycosylation with site-directed mutagenesis. We found that *N*-glycosylation of MCHR1 at Asn^{23} is important for cell surface expression, ligand binding and signal transduction.

2. Materials and methods

2.1. cDNA and mutagenesis

The incorporation of a sequence encoding the Flag epitope tag before the first methionine in rat MCHR1 was performed by PCR. The purified full-length cDNA of MCHR1 was subcloned into pcDNA3.1 (Invitrogen, CA, USA). The generation of the construct was confirmed by sequencing analysis. Oligonucleotide-mediated site-directed mutagenesis was performed using a Quickchange site-directed mutagenesis kit (Stratagene, CA, USA). The oligonucleotides were designed to replace the asparagine residues at positions Asn^{13}, Asn^{16} and Asn^{23} with glutamine (Q) residues. Single-site mutations

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Abbreviations: GPCR, G-protein-coupled receptor; MCH, melanin-concentrating hormone; MCHR1, melanin-concentrating hormone receptor 1; Flag-MCHR1, amino-terminal Flag-tagged MCHR1; FBS, fetal bovine serum; DMEM, Dulbecco's modified Eagle's medium; Endo H, endoglycosidase H; PNGase F, peptide *N*-glycosidase F; PBS, phosphate-buffered saline; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; FITC, fluorescein isothiocyanate

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were first constructed. To elucidate the cumulative effects of a lack of glycosylation at multiple sites, three double receptor mutants, N13,16Q, N13,23Q, and N16,23Q, were constructed. In the triple mutant, all three potentially glycosylated asparagine residues, N13, 16, 23Q, were replaced with glutamine residues.

2.2. Cell culture and transfection

DNA was mixed with LipofectAMINE transfection reagents (Life Technologies, MD, USA), and the mixture was diluted with Opti-MEM and added to 60–70% confluent HEK293T cells plated on 100-mm dishes [13]. The transfected cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS). Cell membranes were prepared from the cells for immunoprecipitation and Western blotting, and radioligand binding assay, 48 h after transfection. For the calcium influx assay, FACScan flow cytometric analysis and immunocytochemistry, the cells were passaged to 96-well plates, 24-well plates, and cover slips, respectively, 24 h after transfection and cultured for another 24 h at 37°C.

2.3. Western blotting and immunoprecipitation

HEK293T cells were washed with phosphate-buffered saline (PBS), lysed with a rubber policeman in RIPA buffer (50 mM Tris–HCl, pH 8.0, 150 mM NaCl, 1% Triton X-100, 0.1% sodium dodecyl sulfate (SDS), 0.5% deoxycholate, 5 μg/ml leupeptin, 5 μg/ml aprotinin, and 0.5 mM PMSF) for 20 min at 4°C, and the lysates cleared by centrifugation at 18 500×*g* for 20 min at 4°C. Aliquots of the cell lysates, equivalent to 100 μg protein, were treated with 0.7 U of peptide *N*-glycosidase F (PNGase F, Boehringer Mannheim) for 3 h at 37°C. For cleavage with endoglycosidase H (Endo H, Boehringer Mannheim), the membrane preparation (100 μg protein) in 15 μl was mixed with an equal volume of 50 mM sodium acetate (pH 4.8) with 120 μg SDS, and incubated with 10 mU of Endo H for 18 h at 37°C. The reactions were stopped by adding an equal volume of protein gel loading buffer. For immunoprecipitation, an aliquot of the cell membrane lysates (500 μg protein) was precleaned with 30 μl of protein-G-agarose (50% suspension in PBS) at 4°C for 30 min on a rotator. The protein-G-agarose was then removed by centrifuging the lysates at 18 500×*g* for 5 min at 4°C. Subsequently, the precleaned cell membrane lysates were incubated with 2 μg of an anti-Flag M2 antibody (Sigma, St. Louis, MO, USA) in protein-G-agarose for 15 h at 4°C on a rotator. The immune-complex was washed three times with RIPA buffer and once with PBS, and subsequently eluted from the protein-G-agarose by the addition of 30 μl of protein gel loading buffer. Proteins were separated in a 15% SDS–polyacrylamide gel electrophoresis (PAGE) gel, and electro-transferred to a Hybond-P PVDF membrane (Amersham International, Little Chalfont, UK). After blocking with 5% skim milk dissolved in washing buffer (0.2% Tween 20 in Tris–HCl buffered saline), Flag-MCHR1 on the membrane was detected using the M2 antibody (2 μg/ml), followed by horseradish peroxidase-conjugated goat anti-mouse IgG antibody. The reactive bands were visualized with ECL substrates (Amersham International).

2.4. FACScan flow cytometric analysis for cell surface receptors

Transfected HEK293T cells in 24-well plates were fixed with 1% paraformaldehyde for 10 min at room temperature, and incubated with 8 μg/ml anti-Flag M2 in PBS containing 20% FBS for 1 h. The cells were washed three times with PBS and incubated with 10 μg/ml fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG secondary antibody for 1 h. The cells were collected from the wells with 5 mM EDTA and analyzed using a FACScan flow cytometer (Becton Dickinson Immunocytometer Systems, Inc.). Cells were gated by light scatter or exclusion of propidium iodide, and 10 000 cells were acquired for each time point. The mean fluorescence of all cells, minus the mean fluorescence of the cells with only the FITC-conjugated second antibody, was used for the calculations.

2.5. Immunofluorescence microscopy

Transfected HEK293T cells were fixed for 10 min in 3.7% paraformaldehyde in PBS. After two rinses with PBS, the cells were transferred, either with or without permeabilization (in 0.05% Triton X-100 in PBS for 15 min), into blocking solution (20% goat serum in PBS) for 30 min [13], and then incubated with 8 μg/ml anti-Flag M2 antibody for 1 h. The anti-Flag M2 antibody was detected using an FITC-conjugated goat anti-mouse IgG secondary antibody (Alexa Fluor 488, Molecular Probes). Fluorescent imaging was obtained with TE300/Radiance2000 confocal microscopy (Bio-Rad).

2.6. Radioligand binding

Transfected HEK293T cells in suspension (8×10^5 cells) were incubated with 1700 Bq [^{125}I] (Phe^{13}, Tyr^{19}) MCH (Amersham International) in the absence (total binding) or presence (non-specific binding) of 1 μM non-labeled MCH (Peptide Institute, Osaka, Japan) in 0.3 ml DMEM supplemented with 50 mM HEPES and 2% BSA for 2 h at 4°C with gentle shaking. Subsequently, the entire contents of the cells were filtered using a GF/C filter presoaked with 0.3% polyethyleneimine and the filter was washed three times with 1 ml of PBS. The radioactivity on the filter was counted using a γ-counter. Specific binding was defined as the difference between the total binding and the non-specific binding.

2.7. Measurement of calcium influx

Transfected HEK293T cells seeded on black-walled 96-well plates (Becton Dickinson, NJ, USA) were loaded for 1 h at 37°C with a non-wash calcium dye (Calcium assay kit, Molecular Device, CA, USA) in Hanks' balanced salt solution containing 20 mM HEPES (pH 7.5). The level of $[Ca^{2+}]_i$ was then detected using a Flexstation imaging plate reader (Molecular Device) over a 150-s stimulation period at each concentration of MCH. Data were expressed as fluorescence (arbitrary units) versus time. The EC_{50} values for MCH were calculated by curve fitting with the program SOFTmax Pro (Molecular Device).

3. Results

3.1. Western blot analysis of Flag-MCHR1 and the mutants

Previously we used an anti-MCHR1 antibody against the carboxy-terminal region (Bachem, Germany) to identify MCHR1 in the transfected cells [20], but this commercial antibody was not suitable for Western blotting. Therefore, we transfected amino-terminal Flag-tagged MCHR1 (Flag-MCHR1) into HEK293T cells in order to identify the full-length MCHR1. In the control calcium influx assays, non-tagged MCHR1 and Flag-MCHR1 were found to have similar EC_{50} values for MCH (data not shown), indicating that

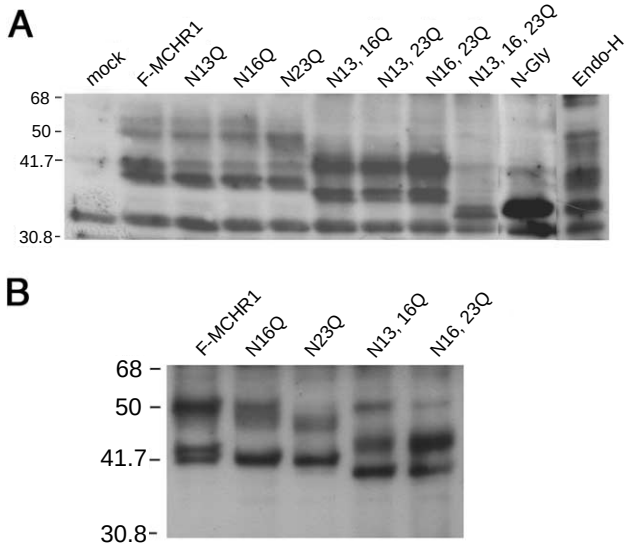

Fig. 1. Expression of Flag-MCHR1 and mutant receptors in HEK293T cells. A: Transfected cells were lysed and 50 μg total protein was separated by 15% SDS–PAGE, transferred to PVDF membranes and immunoblotted with the anti-Flag M2 antibody. The cell extracts transfected with Flag-MCHR1 were treated with peptide *N*-glycosidase F (N-Gly) or Endo H. The lowermost band is a non-specific one which reacted with the anti-Flag M2 antibody. B: Immunoprecipitation of cell lysates by the anti-Flag M2 antibody. N13Q and N13,23Q showed a similar molecular weight to N16Q and N16,23Q, respectively.

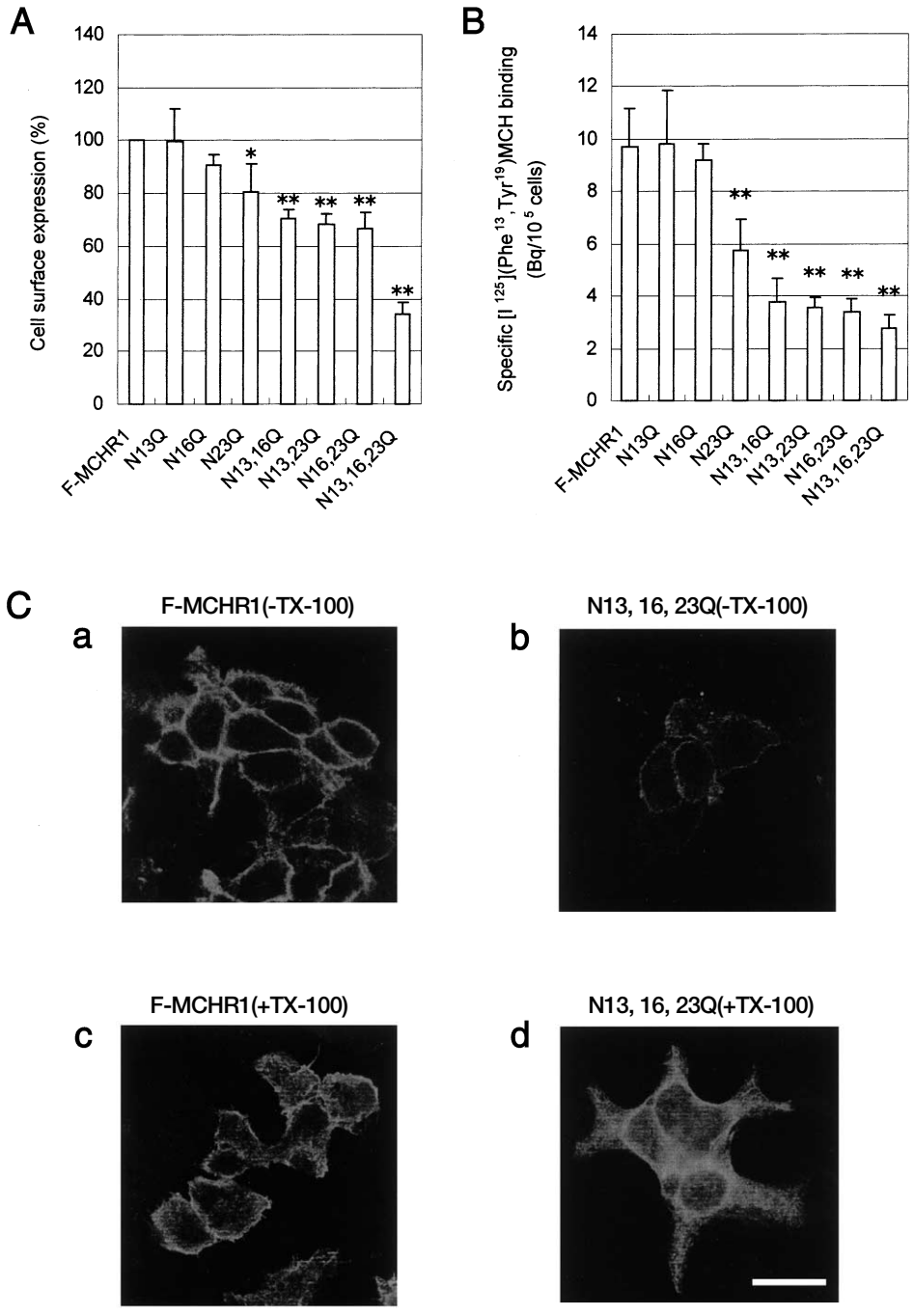

Fig. 2. Cell surface expression and specific radioligand binding of Flag-MCHR1 and mutant receptors. The results are means ± S.E.M. of three independent experiments. $*P < 0.05$, $**P < 0.01$ compared with Flag-MCHR1 by students *t*-test. A: Flow cytometric analysis of the cell surface expression of the mutant receptors using the anti-Flag M2 antibody. The percentage with respect to Flag-MCHR1 (100%) was calculated for each mutant. B: Specific radioligand binding of [^{125}I] (Phe^{13}, Tyr^{19}) MCH to transfected cells in suspension. Non-specific binding was quantified in the presence of 1 μM unlabeled MCH. C: Cellular localization of Flag-MCHR1 and N13,16,23Q by immunofluorescence staining with the anti-Flag M2 antibody and confocal microscopy. The cell surface expression was compared using transfected non-permeabilized cells (a,b) and permeabilized cells (c,d). Vector-transfected cells incubated with the anti-Flag M2 antibody showed no significant staining (data not shown). Bar = 10 μm.

the addition of the Flag tag did not affect the receptor function. Next Flag-MCHR1 was analyzed by Western blotting using an anti-Flag M2 antibody. The result, shown in Fig. 1A, exhibited several characteristic immunoreactive bands of approximately 35–65 kDa. After enzymatic deglycosylation of Flag-MCHR1 expressed in HEK293T cells with PNGase F, a shift to distinct lower molecular weight bands was observed (Fig. 1A). This indicates the presence of oligosaccharides that were modified in the Golgi (complex or hybrid type) in MCHR1. Moreover, glycosylation of MCHR1 by Endo H-sensitive oligosaccharides modified in the ER (high mannose type) was also observed although there were several MCHR1 bands in the Western blotting.

The predicted amino acid sequence of MCHR1 reveals

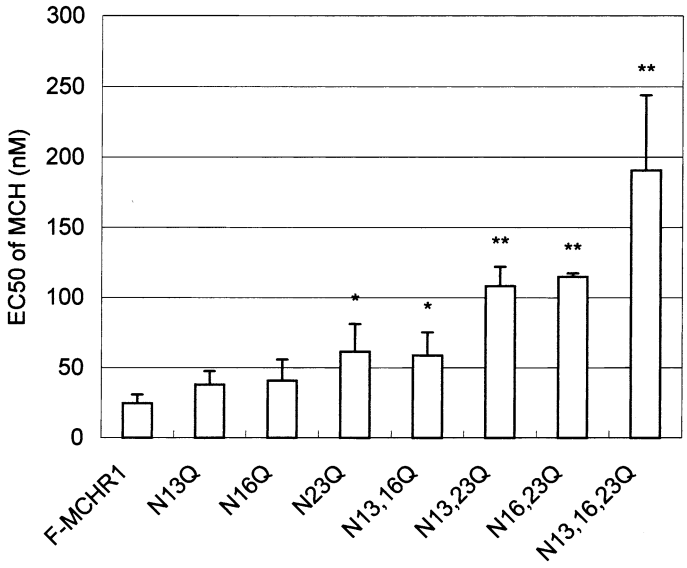

Fig. 3. MCH-stimulated calcium influx in HEK293T cells expressing Flag-MCHR1 or the mutant receptors. The EC_{50} values are means ± S.E.M. of at least three independent experiments.

three potential sites for *N*-glycosylation at Asn^{13}, Asn^{16} and Asn^{23} which are part of consensus sequence (-NXS/T-) [7]. To determine whether these sites are glycosylated, we constructed three MCHR1 mutants in which each of the three asparagine residues was replaced by a glutamine residue. Western blot analysis revealed that mutated receptors at N13Q and N16Q had similar characteristics to those of the wild type Flag-MCHR1, except that the appearance of the 42-kDa band was decreased, while N23Q produced different features, where the uppermost bands were lost (Fig. 1B). These findings indicate that all three potential *N*-linked glycosylation sites in MCHR1 were glycosylated, but that there was greater glycosylation at Asn^{23} compared with Asn^{13} or Asn^{16}. This was further demonstrated by the immunoprecipitation products, in that N23Q migrated faster than N16Q (Fig. 1B). All the double mutants, N13,16Q, N13,23Q and N16,23Q, had reductions of the apparent lower molecular weight bands compared with those of Flag-MCHR1 (Fig. 1A). After immunoprecipitation, the uppermost band in N16,23Q was decreased, while the band was clearly present in N13,16Q (Fig. 1B). Finally, the N13,16,23Q receptor, in which all three glycosylation sites were mutated, revealed the same molecular mass as the deglycosylated product after PNGase F treatment (Fig. 1A). This shows that N13,16,23Q was non-glycosylated MCHR1.

3.2. Cell surface expression of MCHR1 mutant receptors and ligand-binding properties

Cell surface expression of Flag-MCHR1 and the mutant receptors was quantified by FACScan flow cytometric analysis of Flag expression, as shown in Fig. 2A. No significant change was detected in the cell surface expression of the single mutants, N13Q and N16Q, whereas the expression of N23Q was significantly decreased by 25%. The expression of the double mutant receptors N13,16Q, N13,23Q and N16,23Q at the cell surface were decreased by 30–35%, and there were no significant differences among them. In the non-glycosylated mutant, N13,16,23Q, the expression was dramatically decreased by 62%.

Analysis of Flag localization in non-permeabilized cells by confocal microscopy demonstrated that both Flag-MCHR1 and the mutant receptor N13,16,23Q were distinctly localized to the plasma membrane. However, the relative intensity of the membrane localization appeared to be reduced in the N13,16,23Q mutant compared with Flag-MCHR1 (Fig. 2C). This is consistent with the analysis by flow cytometry described above. In permeabilized cells, the N13,16,23Q mutant showed a distinct perinuclear labeling pattern, while N23Q and all the double mutants were localized to the plasma membrane but not to the perinuclear region (data not shown).

To examine whether the expression levels were responsible for the binding of the mutant receptors, we compared the ligand binding levels of Flag-MCHR1 and the mutant receptors. In cells expressing N23Q, specific binding of [^{125}I] (Phe^{13}, Tyr^{19}) MCH was significantly reduced by 40%, although the radioligand binding in N13Q and N16Q was unchanged (Fig. 2B). The specific radioligand binding in all the double mutants was reduced by 60% compared with that of Flag-MCHR1. Finally, even in the total absence of the consensus *N*-glycosylation sites, N13,16,23Q still bound MCH, but the specific binding was exclusively decreased by 72%.

3.3. Effect of glycosylation on MCH-induced calcium influx

The mutant receptors were further characterized for their abilities to cause calcium influx after stimulation with MCH (Fig. 3). Both N13Q and N16Q produced slightly higher EC_{50} values for MCH in calcium influx, while N23Q had more than two-fold higher EC_{50} values for MCH compared with that of Flag-MCHR1 (23 nM versus 58 nM MCH for Flag-MCHR1 and N23Q, respectively). Interestingly, the double mutant N13,16Q had a similar response to the single mutant N23Q, whereas the other double mutants, N13,23Q and N16,23Q, had higher EC_{50} values than that of N13,16Q (108 nM versus 58 nM MCH for N13,23Q and N13,16Q, respectively). The non-glycosylated mutant, N13,16,23Q showed eight-fold higher EC_{50} values (190 nM MCH for N13,16,23Q). Thus, among the double mutants, the potency in MCH-induced calcium

influx appears not to be relative to the extent of cell surface expression and radioligand binding receptor glycosylation (Fig. 2A,B). The single mutants, N13Q, N16Q, and N23Q, and the double mutant N13,16Q did not alter the maximal response of the cumulative fluorescence units caused by 3 μM MCH. However, in N13,23Q, N16,23Q and N13,16,23Q, the maximal response was significantly decreased by 30%, 35%, and 45%, respectively.

4. Discussion

The importance of *N*-linked glycosylation for expression and function has been studied for many GPCRs. Most of the GPCRs of the large rhodopsin subfamily that includes adrenergic receptors and a number of peptide receptors have relatively short extracellular amino-terminals with one or more putative glycosylation sites, but the role of the *N*-glycosylation is somewhat variable. In some [21], glycosylation of at least one site is required for efficient cell surface expression and signal transduction, while in others [22–25], glycosylation at specific residues is crucial for correct delivery to the cell surface and/or signal transduction. In contrast, the non-glycosylated V2 vasopressin receptor was shown to be fully active for ligand binding and signal transduction [26].

Our approach involved mutagenesis of the MCHR1 receptor cDNA to delete the glycosylation sequences in the amino-terminal region, and testing of the resulting molecules for their interaction with the ligand and signal transduction in the transfection system. This study has demonstrated that all of the three potential glycosylation sites of the MCHR1 receptor are glycosylated, and that the sites are not equally relevant for cell surface expression, ligand binding, and MCH-stimulated calcium influx of MCHR1. Single mutation of the asparagines at positions 13 or 16 did not impair the level of cell surface expression of the receptor, radioligand binding or the ability for MCH-mediated calcium influx. Therefore, the carbohydrate moiety at each single glycosylation site at 13 or 16 is not absolutely required for active MCHR1 on the cell surface. Conversely, substitution of Asn^{23} significantly lowered the cell surface expression, ligand binding, and potency for MCH-stimulated calcium influx compared with Flag-MCHR1, indicating that the glycosylation at Asn^{23} is required for optimal expression and function of MCHR1 on the cell surface.

The importance of the glycosylation at Asn^{23} is also indicated by the results of the other mutants. The double mutant N13,16Q exhibited reduced glycosylation compared with the single mutant N23Q in Western blotting (Fig. 1A,B), and there were significant differences between them in both the cell surface expression and radioligand binding (Fig. 2A,B, $P < 0.05$, respectively). However, no difference in the ability to cause calcium influx was observed between in N23Q and N13,16Q (Fig. 3). We created other mutants in which the asparagine residues were replaced by alanine (N23A, N13,16A). These gave similar EC_{50} values for MCH in calcium influx to N23Q and N13,16Q, respectively (data not shown), making it less likely that the replacement of asparagine with glutamine had a significant influence on the functional activity of MCHR1. The double mutants N13,16Q, N13,23Q and N16,23Q showed a similar phenotype in terms of the cell surface expression and radioligand binding (Fig. 2A,B). However, the double mutants with the amino acid replacement at Asn^{23} (N13,23Q and N16,23Q) had a lower potency to cause MCH-stimulated calcium influx than that of N13,16Q (Fig. 3). Again, these results suggest the functional importance of glycosylation at Asn^{23} for signal transduction in MCHR1. This has also been observed in other GPCRs. For example, rhodopsin is glycosylated at Asn^{2} and Asn^{15}, and mutations at or near Asn^{2} had little effect on cell surface expression and G_T activation [22]. In contrast, mutations near Asn^{15} resulted in poor activation of signal transduction. In the secretin receptor, among single mutations at the five potential glycosylation sites, only the Asn^{72} mutation was found to impair the biological activity of the receptor [23].

Previous studies have demonstrated that the loss of carbohydrate moieties associated with membrane receptors led to reduced cell surface expression [21,24,27,28]. In agreement with these reports, progressive disruption of the glycosylation sites of MCHR1 caused a progressive reduction in the cell surface expression as reflected in the FACScan flow cytometric analysis (Fig. 2A). In membrane-permeabilized cells, non-glycosylated MCHR1, N13,16,23Q, was distinctly expressed in the perinuclear region (Fig. 2C). It appears that the glycosylation of MCHR1 plays a role in its correct translocation through the ER and Golgi apparatus to the plasma membrane [27,28]. However, elimination of one or two of the glycosylation sites did not impair the trafficking of the receptor, and thus all the other MCHR1 mutants were correctly delivered to the plasma membrane as visualized by confocal microscopy. This suggests that glycosylation of at least one site may be sufficient for normal intracellular trafficking of MCHR1. This is consistent with the human VIP 1 and secretin receptors [23,24]. Thus, the reduced membrane expression observed in the other mutants (N23Q and the double mutants) is not due to changes in the delivery, but might rather be related to changes in the structure of the receptor. These conformational changes may result in the lower potency for ligand binding and MCH-stimulated calcium influx. It is possible that the amount of sugar moieties is not sufficient to constrain the amino acid residues of the amino-terminus in a conformation that is optimal for MCH binding. Further physical studies with nuclear magnetic resonance spectroscopy or X-ray crystallography will be required to resolve the three-dimensional structure of the MCHR1–MCH complex and the role of the sugar moieties.

In conclusion, all of the potential *N*-glycosylation sites, Asn^{13}, Asn^{16} and Asn^{23}, in MCHR1 are glycosylated. *N*-glycosylation of MCHR1, particularly at Asn^{23}, seems to play roles in the cell surface expression, ligand binding, and signal transduction of MCHR1 to display the full activity of the receptor. The mechanism of how glycosylation at the amino-terminus can effect events throughout the receptor, including events at distal regions such as the carboxy terminus, is unclear but indicates a further complexity through which the cell can regulate its responses to GPCR activation.

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